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(57) Abstract

The present invention relates to the use of phospholipase C–γ1 gene for the diagnosis, prognosis and development of treatments of bipolar disorder and/or lithium response. There is provided a method for the identification of patient with susceptibility to bipolar disorder and/or lithium response, which comprises the steps of: a) obtaining a nucleic acid sample of the patient; and b) determining allelic variants of phospholipase C–γ1 gene present in the sample, and wherein certain allelic variants are indicative of a risk of pipolar disorder and/or lithium response.

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# ALLELIC VARIANTS OF THE PHOSPHOLIPASE C-γ1 GENE ASSOCIATED WITH BIPOLAR DISORDER

## BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to the use of phospholipase C-γ1 gene for the diagnosis, prognosis and development of treatments of bipolar disorder and also to be associated with lithium response.

(b) Description of Prior Art

Bipolar disorder (BD) is a major psychiatric condition that affects up to 1% of the general population and results in episodes of mania and depression. Lithium has been used in the prophylaxis and treatment of BD for almost half a century, and remains the first-choice therapy for preventing recurrences (Schou M., *Arch. Gen. Psychiatry*, 1997; **54**:9-13; discussion 14-5). Although lithium is considered specific for the treatment of BD, with no comparable effect in other psychiatric disorders, its effectiveness varies widely. There is compelling evidence that lithium is more effective in forms of BD characterized by typical symptomatology and the absence of comorbidity. There is also evidence that responders and nonresponders to lithium treatment differ in certain neuroendocrine responses involving the serotonergic and endorphin systems. In addition, family studies indicate a higher recurrent risk for bipolar disorder among relatives of patients who respond well to lithium treatment (Grof P et al., *J. Affect. Disord.*, 1994; **32**:85-95). Taken together, these findings suggest that response to lithium prophylaxis

may help define a distinct bipolar phenotype with less genetic heterogeneity.

The mechanism by which lithium acts is not exactly known. Recent findings indicate that lithium may stabilize mood by acting at the phosphoinositide second messenger system (Manji HK et al., *Arch. Gen. Psychiatry.*, 1995; **52**:531-543). Cellular responses mediated by inositol phospholipides are involved in many brain processes. They are initiated by a phospholipase C (PLC) isozyme after activation by a membrane receptor that can be coupled to a G protein, protein tyrosine kinase or several lipid-derived second messengers such as arachidonic acid. Lithium is thought to inhibit the enzyme inositol monophosphatase, leading to a reduction in the availability of inositol (Manji HK et al., *Arch. Gen. Psychiatry.*, 1995; **52**:531-543).

It would be highly desirable to be provided with a gene and its uses thereof for the diagnosis, prognosis and development of treatments of bipolar disorder.

## SUMMARY OF THE INVENTION

One aim of the present invention is to provide the use of phospholipase C-γ1 gene for the diagnosis, prognosis and development of treatments of bipolar disorder and also to be associated with lithium response.

In accordance with the present invention, we present evidence suggesting that bipolar patients with an excellent response to lithium prophylaxis have a higher frequency of a polymorphism located in the gene coding for the γ-1 isozyme of phospholipase C (PLCG1) on chromosome 20.

In accordance with the present invention there is provided a method for the identification of patient

with susceptibility to bipolar disorder and/or prediction of lithium response, which comprises the steps of:

a) obtaining a nucleic acid sample of the patients; and

b) determining allelic variants of phospholipase C-γ1 gene present in said sample, and wherein certain allelic variants are indicative of a risk of bipolar disorder and/or lithium response.

In accordance with the present invention, certain allelic variants of the phospholipase C-γ1 gene relative to the wild type phospholipase C-γ1 gene are indicative of bipolar disorder. Preferably, and in accordance with the present invention, the allelic variants include, without limitation, PLCG1 dinucleotide repeats of about 174, 176 and 178 bp in length respectively.

In accordance with the present invention, the bipolar disorder may be a mood disorder.

The patient may be responding to lithium treatment.

In accordance with the present invention there is also provided a non-human mammal model for the phospholipase C-γ1 gene, whose germ cells and somatic cells are modified to express at least one allelic variant of the phospholipase C-γ1 gene or are phospholipase C-γ1 gene knockout and wherein the allelic variant or knockout of the phospholipase C-γ1 gene being introduced into the mammal, or an ancestor of the mammal, at an embryonic stage.

In accordance with the present invention there is also provided a method for the screening of therapeutic agents for the prevention and/or treatment of bipolar disorder, which comprises the steps of:

a) administering the therapeutic agents to the non-human mammal of the present invention or bipolar disorder patients; and

b) evaluating the prevention and/or treatment of development of bipolar disorder in the mammal or the patients.

In accordance with the present invention there is also provided a method of stratifying bipolar disorder patients based on the allelic variants of the phospholipase C-γ1 gene for clinical trials purposes, which comprises:

a) obtaining a nucleic acid sample of the patients; and

b) determining allelic variants of the phospholipase C-γ1 gene, wherein patients are stratified with respect to their allelic variants and wherein certain allelic variants are indicative of bipolar disorder and/or lithium response.

In accordance with the present invention there is also provided a method to identify gene parts of or interacting with a biochemical pathway affected by phospholipase C-γ1 gene, which comprises the steps of:

a) designing probes and/or primers using phospholipase C-γ1 gene and biological samples with the probes and/or primers; and

b) evaluating the identified gene role in bipolar disorder patients and/or lithium response.

## BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 illustrates a graph of the PLCG1 dinucleotide repeats allele frequency distribution in patients and controls.

## DETAILED DESCRIPTION OF THE INVENTION

Several studies have indicated that patients with bipolar disorder (BD) who respond well to lithium prophylaxis constitute a biologically distinct subgroup. Lithium is thought to stabilize mood by acting at the phosphoinositide cycle. We have investigated a polymorphism located in the gene (PLCG1) that codes for a γ-1 isozyme of phospholipase C, an enzyme that plays an important role in the phosphoinositide second messenger system.

**Methods**

A population-based association study and a family-based linkage study were carried out. Only patients who were considered excellent responders to lithium prophylaxis were studied. Response to lithium was evaluated prospectively with an average follow-up of 14.4 ± 6.8 years. The PLCG1 polymorphism was investigated in 138 excellent lithium responders and 163 controls. In addition, the segregation of this marker was studied in 32 families ascertained through lithium responsive bipolar probands.

**Findings**

The allele distributions between lithium responsive bipolar patients and controls were different, with a higher frequency of one of the PLCG1 polymorphisms in bipolar patients ($\chi^2$ =8.09; p=0.004). This polymorphism, however, confers only a small risk as estimated by the odds ratio (1.88, CI 1.19-3.00). Linkage studies with the same marker yielded modest, additional support for the involvement of this gene in the pathogenesis of lithium responsive BD.

**Interpretation**

Our results provide preliminary evidence that a PLC isozyme may confer susceptibility to lithium responsive bipolar disorder, probably accounting for a fraction of the total genetic variance. Whether this polymorphism is implicated in the pathogenesis of BD or in the mechanism of lithium response remains to be determined.

**PATIENTS AND METHODS**

***Association study sample***

Patients with bipolar disorder were recruited from six centres that collaborate in the International Group for the Study of Lithium (IGSLi) (see below). These patients have been followed within specialized lithium clinics with the objective of systematically and prospectively assessing their response to lithium prophylaxis. The diagnoses were based on Schedule for Affective Disorders and Schizophrenia (SADS-L) interview and Research Diagnostic Criteria (RDC). In order to be included in the study, all patients had to meet stringent criteria of excellent lithium response, described previously (Grof P et al., *J. Affect. Disord.*, 1994; **32**:85-95). All diagnostic and treatment information was reviewed by a panel of experienced psychiatrists in a blind fashion. To further insure uniformity of diagnoses in six different countries, all subjects were subsequently assessed by the same senior clinician. All patients were Caucasians of Western and Central European descent. A total of 138 patients were included: 68 from Canada, 23 from Germany, 20 from the Czech Republic, 17 from Sweden, 7 from Denmark, and 3 from Austria. The mean (± standard deviation) age of

onset was 27.6 (± 9.9) years. The number of the illness episodes prior to lithium treatment was 8.2 (± 10.1). Patients have been stabilized on lithium monotherapy for 14.4 (± 6.8) years.

Control subjects for this study were 128 psychiatrically unaffected individuals screened with a SADS-L interview and RDC criteria. Controls were collected in similar fashion in Canadian and European centres, and consisted of healthy married-in individuals from the linkage study, hospital staff and normal volunteers. An additional 35 control subjects were included without assessment of psychiatric status. All cases and controls were of similar ethnic background and matched for geographical origin whenever possible. The mean age (± standard deviation) and sex ratio (M:F) were 50.0 (± 14.4) years, 0.87 and 0.86, 51.45 years (±14.8) for patients and controls respectively.

***Linkage study sample***

Thirty two (32) of the above Canadian probands had families available for linkage analysis. Relatives in these families were personally interviewed by two psychiatrist in a blind fashion using SADS-L interviews. Best-estimate consensus diagnoses were made blindly by a panel of psychiatrists taking into account all available sources of information. Relatives were considered affected if they met RDC criteria for bipolar disorder, recurrent schizoaffective disorder or recurrent major depression with the additional criterion of functional impairment during depressive episodes. The families comprised a total of 629

individuals, of whom 224 (95 affected) were interviewed and sampled.

All individuals included in the association and linkage studies provided written informed consent.

Genomic DNA was extracted by a standard method from venous blood samples. The PLCG1 polymorphism was originally identified by searching a cosmid isolated from a clone containing the PLCG1 cDNA. This is an untranslated CA repeat with 15 detected alleles ranging from 150 to 184 bp (Rothschild CB et al., *Genomics*, 1992; **13**:560-564). PCR was carried out in a total volume of 12.5 ul containing 40 ng genomic DNA; 125 ng of primers PLCpr1 (5'-AACCAGTCTGCTCTTCCGGTG-3', SEQ ID NO:1) and PLCpr2 (5'-CTGCCTTCAACTGATCTCAATGG-3', SEQ ID NO:2); 200 uM each of dGTP, dCTP, and dTTP; 25 uM dATP; 1.5 uCi [35S]DATP; 0.5 units of Taq DNA polymerase (Bio/Can Scientific); and 2.0 ul of 10X buffer (Bio/Can Scientific) with $MgCl_2$ included in the final concentration of 1.5 mM. Samples were over-laid with mineral oil and processed throughout 35 cycles of denaturation at 94°C, annealing at 56°C, and elongation at 72°C, followed by a final elongation period of 72°C. PCR products were analyzed on a 6% denaturating polyacrylamide gel (38:2 acrylamide:bisacrylamide). Samples were run for a period of 2h in a vertical electrophoresis gel apparatus (Life Technologies). Gels were dried and exposed to x-ray films for 48 to 72h at room temperature. All marker determinations were made blind to the clinical diagnoses. Autoradiographs were read and interpreted independently by two different raters, and both readings yielded identical results.

The presence of an association was investigated using $\chi^2$ and odds ratio tests by standard methods. Parametric linkage analysis was carried out using the MLINK program of the FASTLINK package. Three major genetic models were explored in order to maximize the evidence for linkage. These models were: a) dominant [allele frequency (q) 0.012, male penetrance (fM) 0.4, female penetrance (fF) 0.7, and normal penetrance 0.005 for males (fM0) and 0.009 for females (fF0)]; b) intermediate [q 0.024, fM 0.4/0.2, fF 0.7/0.35, fM0 0.005, fF0 0.009]; c) recessive [q 0.16, fM 0.35, fF 0.65, fM0 0.005, fF0 0.009]. The parameters for the recessive model were based on segregation analysis of this sample (Alda M et al., *J. Affect. Disord.*, 1997; **44**:153-157). In order to estimate the significance of the results, simulations were carried out using the SIMULATE and SLINK programs. Nonparametric linkage analysis was conducted using the SimIBD program which calculates a simulation-based nonparametric statistic that provides a powerful test for linkage. Corrections for multiple testing were made for subgroup comparisons using Bonferrroni's procedure (which is indicated by *).

**RESULTS**

Allele distributions in Canadian and European individuals were similar, both for cases ($\chi^2$=1.12, p=0.29), and controls ($\chi^2$=0.170, p=0.68). This allowed pooling of the Canadian and European samples (patients as well as controls) for all comparisons.

The distribution of alleles in patients and controls is shown in Table 1.

Table 1

Allele frequency distribution in lithium responder bipolar patients and controls

| | | Allele Frequency (%) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *N* | *PLC G1/1* | *PLC G1/2* | *PLC G1/4* | *PLC G1/5* | *PLC G1/6* | *PLC G1/7* | *PLC G1/8* | *PLC G1/9* | *PLC G1/10* | *PLC G1/11* | *PLC G1/12* | *PLC G1/13* | *PLC G1/16* | *PLC G1/17* | *PLC G1/18* |
| **Controls** | 163 | 0.6 | 0.9 | 5.0 | 12.6 | 6.3 | 5.0 | 2.8 | 1.6 | 51.9 | 0.6 | 1.3 | 6.0 | 0.3 | 4.1 | 0.9 |
| **Patients** | 138 | 1.5 | 0.4 | 7.4 | 21.3 | 7.4 | 4.0 | 2.9 | 0.0 | 43.0 | 0.7 | 0.0 | 2.9 | 1.1 | 5.1 | 2.2 |
| **Total** | 301 | 1.0 | 0.7 | 5.8 | 16.6 | 6.8 | 4.6 | 0.8 | 0.8 | 47.8 | 0.7 | 0.7 | 4.4 | 0.7 | 4.6 | 1.5 |

A significant difference was observed in the overall allele distribution between patients and controls ($\chi^2$ = 27.06; p=0.04) with allele PLCG1/5 being considerably more frequent among patients than controls ($\chi^2$ =8.09; p=0.004). In order to take into account possible instability of the dinucleotide repeat, we investigated if the difference between groups persisted within the bounds of alleles with ± 1 and ± 2 repeats. Indeed, patients differed from controls when these alleles were pooled together (PLCG1/5±1: $\chi^2$=10.35; p=0.0026* and PLCG1/5±2: $\chi^2$=8.10; p=0.0088*).

Parametric linkage results using the PLCG1 marker provided negative maximum LOD scores when results from all families were considered (results not shown). As previous findings suggested that the study of unilineal pedigrees might provide an advantage to overcome part of the complexity of bipolar disorder, the data were further analyzed according to lineality. Among all pedigrees, 13 were clearly unilineal (6 of paternal and 7 of maternal origin). The largest lod score observed among unilineal families was 1.45 (p=0.004) under the dominant model. There was no difference between maternal and paternal pedigrees. Nonparametric linkage analysis provided similar results.

**DISCUSSION**

An increasing body of evidence supports the hypothesis that alterations in the phosphoinositide signal transduction system may be implicated in the pathophysiology of bipolar disorder (Manji HK et al., *Arch. Gen. Psychiatry.*, 1995; **52**:531-543). Studies of postmortem brain samples from patients with BD and

suicide victims with major depression have shown a marked reduction in phosphatidylinositol hydrolysis by G protein coupled to phospholipase C stimulation, when compared to normal controls. Furthermore, recent magnetic resonance spectroscopy studies have indicated that lithium reduces myoinositol levels in critical brain regions in bipolar patients. The phosphoinositide-specific PLC γ-1 isozyme is present in neurons; studies in rats have found it highly localized in the hippocampus and basal ganglia. PLC g-1 is activated by a number of receptors that promote phosphorylation of the enzyme, usually via protein tyrosine kinase, resulting in the hydrolysis of phosphatidylinositol 4,5-biphosphate, which produces two intracellular messengers: diacylglycerol and inositol 1,4,5-triphosphate (Rhee SG et al., *J. Biol. Chem.*, 1997; **272**:15045-15048). It is therefore possible that PLCG1 constitutes part of the total genetic variability predisposing to BD.

The finding of an association between PLCG1 and BD in our study suggests that this polymorphism is in linkage disequilibrium with the sequence/gene that confers susceptibility to BD. This interpretation is supported by the observation that, compared to controls, patients have a higher frequency of alleles within the bounds of ± 2 repeats from the associated allele. Furthermore, microsatellite repeats are usually not transcribed, and therefore are commonly regarded as non-functional, unlikely to confer susceptibility to BD.

However, positive findings in association studies are usually open to alternative interpretations which we must consider in this case as well. It is possible, for example, that the microsatellite marker tested in this study by itself confers certain susceptibility to BD. There is increasing evidence that certain tandem repeat sequences, such as VNTRs do influence gene transcription, thereby influencing phenotype expression (Bennett ST et al., *Annu. Rev. Genet.*, 1996; **30**:343-370). In addition, comparative genomic studies have indicated that repeat arrays and genomic locations of dinucleotide repeats are highly conserved, suggesting that CA repeats may have a functional significance (Deka R et al., *Genomics*, 1994; **22**:226-230). Their role has been hypothesized to be, among others, related to the regulation of gene transcription.

Finally, at this point we cannot yet completely exclude the possibility that the finding is spurious. It is conceivable that our findings reflect problems such as admixture or population stratification effects, rather than a 'true' genetic association. We consider this interpretation unlikely for the following reasons. Although collected in different geographic locations, the European and Canadian patients included in this study share a common background. In addition, when Canadian and European data were compared, both stratified and not stratified for clinical status, no differences in allele frequency distribution were found.

The results obtained for unilineal families in the parametric analysis, as well as those observed in

the nonparametric study were not significant given the standard criteria employed in linkage studies. However, they could well be regarded as an additional support for the role of PLCG1 in the etiology of BD if we consider these results in conjunction with those obtained in the association study. Defining thresholds of significance in linkage studies of complex traits has been rather difficult (Risch N et al., *Nat. Genet.*, 1996; **12**:351-353). It is clear that in order to avoid false positive results, stringent criteria should be used. This may lead, however, to situations in which samples of "realistic" sizes will not have sufficient power to detect loci that account for a small proportion of the total genetic variability (Risch N et al., *Nat. Genet.*, 1996; **12**:351-353). Considering the genetic and phenotypic complexity of BD, as well as the modest risk attributed to the PLCG1 locus, it is likely that our family sample was not large enough to achieve usual significance criteria. Hence, given the positive association findings, the results of the linkage study, quite unlikely to be observed by chance (p=0.003, p=0.012 in the parametric linkage analysis, and p= 0.089 in the nonparametric linkage analysis) could be regarded as additional evidence that PLCG1 may play a role in the pathogenesis of lithium responsive BD.

Further evidence supporting the involvement of this locus in the etiology of lithium responsive BD will have to come through independent replication. Consistency between studies is certainly an important way to validate findings. In this context, it is important to note that the patient cohort used in this study was carefully selected according to a systematic

and prospective evaluation of lithium response. Therefore, this locus could be related to the capacity to improve with lithium treatment rather than to bipolar disorder. Thus, the validity of future attempts at independent replication of these findings will be contingent on lithium response being evaluated according to similar methodology and criteria.

In conclusion, we have found preliminary evidence that a polymorphism at the locus that codes for a PLC isozyme is associated to lithium responsive BD. Whether the locus that confers susceptibility to lithium responsive BD is the (CA)n, another polymorphism/mutation in the PLCG1 gene or another neighboring gene remains to be clarified. We are currently conducting further studies investigating this gene for additional polymorphisms which may be similarly associated to this disorder.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method for the identification of patient with susceptibility to bipolar disorder and/or prediction of lithium response, which comprises the steps of:

a) obtaining a nucleic acid sample of said patient; and

b) determining allelic variants of phospholipase C-γ1 gene present in said sample, and wherein certain allelic variants are indicative of a risk of bipolar disorder and/or lithium response.

2. The method of claim 1, wherein said bipolar disorder is a mood disorder.

3. The method of claim 1, wherein said patient is responding to lithium treatment.

4. The method of claim 1, wherein said allelic variants are selected from the group consisting of PLCG1 dinucleotide repeats between 174 and 178 bp.

5. A non-human mammal model for the phospholipase C-γ1 gene, whose germ cells and somatic cells are modified to express at least one allelic variant of the phospholipase C-γ1 gene or are phospholipase C-γ1 gene knockout and wherein said allelic variant or knockout of the phospholipase C-γ1 gene being introduced into the mammal, or an ancestor of the mammal, at an embryonic stage.

6. A method for the screening of therapeutic agents for the prevention and/or treatment of bipolar disorder, which comprises the steps of:

a) administering said therapeutic agents to the non-human mammal of claim 5 or bipolar disorder patients; and

b) evaluating the prevention and/or treatment of bipolar disorder in said mammal or said patients.

7. A method of stratifying bipolar disorder patients based on the allelic variants of the phospholipase C-γ1 gene for clinical trials purposes, which comprises:

a) obtaining a nucleic acid sample of said patients; and

b) determining allelic variants of the phospholipase C-γ1 gene, wherein patients are stratified with respect to their allelic variants and wherein certain allelic variants are indicative of bipolar disorder and/or lithium response.

8. A method to identify genes part of or interacting with a biochemical pathway affected by phospholipase C-γ1 gene, which comprises the steps of:

a) designing probes and/or primers using phospholipase C-γ1 gene and screening biological samples with said probes and/or primers; and

b) evaluating the identified gene role in bipolar disorder patients and/or in lithium response.

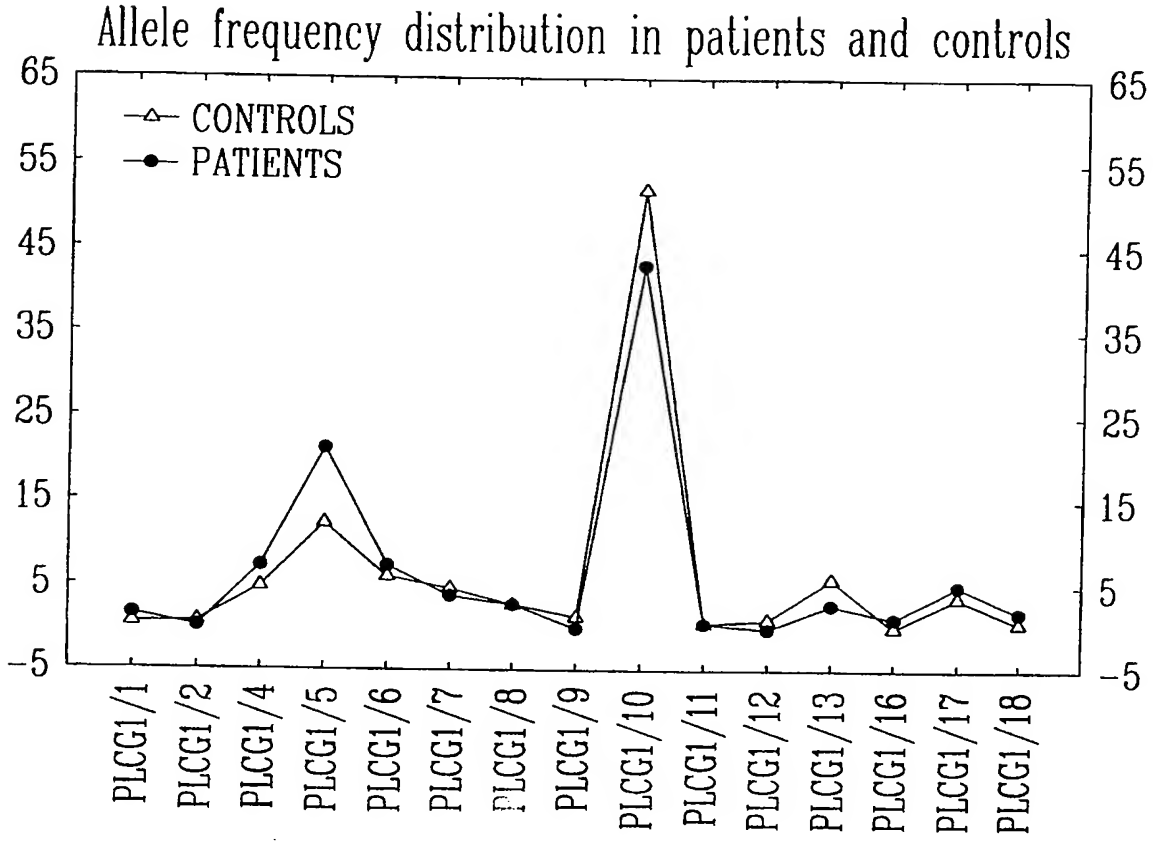


Fig - 1

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<120> ALLELIC VARIANTS OF THE PHOSPHOLIPASE C-γ1 GENE ASSOCIATED WITH BIPOLAR DISORDER

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# INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 A01K67/027 C12N5/10 //C12N15/55

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| A | JOPE R ET AL: "THE PHOSPHOINOSITIDE SIGNAL TRANSDUCTION SYSTEM IS IMPAIRED IN BIPOLAR AFFECTIVE DISORDER" JOURNAL OF NEUROCHEMISTRY, vol. 66, no. 6, June 1996, pages 2402-9, XP002091618 see the whole document --- -/-- | 1 |

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | EBSTEIN R ET AL: "Lithium modulation of second messanger signal amplification in man: inhibition of phosphatidylinositol-specific phospholipase C and adenylate cyclase activity" PSYCHIATRY RESEARCH, vol. 24, no. 1, April 1988, pages 45-52, XP002091619 see the whole document | 1 |

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